



Original Article

Contextualised urinary biomarker analysis facilitates diagnosis of paediatric obstructive sleep apnoea



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ABSTRACT

Background: Intrinsic variance of the urine proteome limits the discriminative power of proteomic analysis and complicates potential biomarker detection in the context of paediatric sleep disorders.

Methods and results: Using a rigorous workflow for proteomic analysis of urine, we demonstrate that gender and diurnal effects constitute two important sources of variability in healthy children. In the context of disease, complex pathophysiological perturbations magnify these proteomic differences and therefore require contextualised biomarker analysis. Indeed, by performing biomarker discovery in a gender- and diurnal-dependent manner, we identified ~30-fold more candidate biomarkers of paediatric obstructive sleep apnoea (OSA), a highly prevalent condition in children characterised by repetitive episodes of intermittent hypoxia and hypercapnia, and sleep fragmentation in the context of recurrent upper airway obstructive events during sleep. Remarkably, biomarkers were highly specific for gender and sampling time as poor overlap (~3%) was observed in the proteins identified in boys and girls across morning and bedtime samples.

Conclusions: As no clinical basis to explain gender-specific effects in OSA or healthy children is apparent, we propose that implementation of contextualised biomarker strategies will be applicable to a broad range of human diseases, and may be specifically applicable to paediatric OSA.

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1. Introduction

Obstructive sleep apnoea (OSA) is a highly prevalent disorder in children (2–3%) characterised by repeated events of partial or complete obstruction of the upper airway during sleep. This frequent condition, which results in recurring episodes of hypercapnia, hypoxaemia and arousal throughout the night, leads to a substantial risk of development of cardiovascular, metabolic and neurobehavioural and cognitive problems [1–6].

The gold standard diagnostic approach for OSA is an overnight sleep study, or polysomnography (PSG). While this approach is reliable, it suffers from problems associated with its implementation in the clinical setting. Indeed, PSG is labour intensive, inconvenient and expensive resulting in long waiting periods and unnecessary delays in diagnosis and treatment. From this perspective, the identification of surrogate biomarkers that reliably diag-

nose OSA would substantially overcome these problems and enable early detection and intervention for this important medical problem.

Urine is a highly desirable biological fluid for diagnostic testing because of its ease of collection and widespread use in clinical laboratories. Biomarker discovery strategies in urine, however, have been hindered by problems associated with reproducibility and inadequate standardisation of proteomic protocols. Despite these concerns, urinary proteomics analyses have been leveraged to identify numerous candidate biomarkers of a broad range of human disorders that have included, but are not limited to, renal disease, diabetes, atherosclerosis, Alzheimer's disease and cancer [7–14]. Furthermore, our previous studies identified 12 candidate urinary biomarkers capable of reliably detecting OSA in a restricted cohort of children [15].

The litmus test for any given biomarker involves validation in large patient populations, a requirement that few biomarkers pass. Indeed, our preliminary attempts to validate our previously identified biomarkers of OSA in a new patient cohort have highlighted the difficult nature of this endeavour. Genetic and environmental variability impose extraordinary heterogeneity on patient populations, and people in general. These factors introduce significant

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‘instability’ into the urinary proteome, making the identification of biomarkers with universal application a daunting challenge [16].

In an attempt to circumvent these problems, we interrogated two important likely sources of variability (gender and diurnal effects) on both the urine proteome and biomarker discovery process of paediatric OSA. To facilitate this process, we developed a rigorous and reproducible proteomics workflow for biomarker discovery based on liquid chromatography–tandem mass spectrometry (LC–MS/MS), an approach that allows for deeper proteome coverage and interrogation of lower-abundance proteins. Our findings demonstrate the presence of dramatic gender and diurnal effects on biomarkers of OSA, suggesting that discovery-based proteomics approaches aimed at identifying biomarkers in a contextualised manner may greatly facilitate our ability to reliably detect human disease.

2. Materials and methods

2.1. Ethics statement

The research protocol was approved by the University of Chicago (protocol 10-708-A-AM011) human research ethics committee. Written informed consent was obtained from the parents and age-appropriate written assent from the children.

2.2. Patient information

Children (ages 2–12 years) clinically referred for evaluation of OSA underwent an overnight polysomnographic evaluation at the University of Chicago Pediatric Sleep Laboratory. Healthy children were recruited from schools or well-child clinics. Exclusion criteria for all subjects included the presence of significant genetic or craniofacial syndromes, diabetes, cystic fibrosis, cancer or treatment with oral corticosteroids, antibiotics or anti-inflammatory medications. All parents completed a detailed intake clinical questionnaire. Height, weight and vital signs were recorded for each child, and body mass index (BMI) z-score was calculated on the basis of Centers for Disease Control and Prevention (CDC) 2000 growth standards (www.cdc.gov/growthcharts) and using online software (www.cdc.gov/epiinfo). A BMI z-score exceeding 1.65 (.95th percentile) was considered as fulfilling criteria for obesity.

2.3. Overnight PSG

All subjects underwent an overnight PSG using standard methods [17]. The PSG studies were scored as per the 2007 American Association of Sleep Medicine guidelines for the scoring of sleep and associated events [18]. The obstructive apnoea–hypopnoea index (AHI) was defined as the number of obstructive apnoeas and hypopnoeas per hour of total sleep time.

2.4. Urine collection

Midstream urine specimens were collected in the evening just before bedtime and as the first void in the morning after awakening. Samples (20 mL) were collected into tubes containing phenylmethylsulphonyl fluoride (PMSF, 2 mM final concentration), and immediately stored at -80°C until analysis.

2.5. Study design

Morning and bedtime urine samples from healthy children ($N = 13$; seven boys and six girls) and children with OSA ($N = 14$; seven boys and seven girls) were collected. Samples were analysed by MS (single injection) and enzyme-linked immunosorbent assay

(ELISA) (in duplicate). Analyses of the resultant proteomics data (Tables S1–S3) were used to assess the reproducibility of the proteomics workflow, identify diurnal- and gender-dependent effects on the urine proteome of healthy children and detect candidate biomarkers of paediatric OSA. See below for details.

2.6. Preparation of soluble urine proteins for MS

Urine (10 mL) was thawed quickly at 37°C , vortexed for 90 s and centrifuged (500g, 4°C) for 5 min. Supernatants were centrifuged at 12,000g, 4°C for 20 min to remove urinary sediment, and incubated with 1 mL ProteinG magnetic beads (Millipore) for 30 min at 20°C . Depletion of immunoglobulin G (IgG) was performed according to the manufacturer’s protocol. IgG-depleted urine samples were precipitated using trichloroacetic acid and deoxycholate (TCA/DOC) as previously described [19,20]. Briefly, urine was supplemented with 0.02% sodium deoxycholate and 20% trichloroacetic acid, and incubated overnight with rocking at 4°C . Proteins were harvested by centrifugation (18,000g for 60 min at 4°C). The protein pellet was washed twice with ice-cold acetone, and reconstituted in 0.1% RapiGest (Waters Corp.), 250 mM ammonium bicarbonate, pH 8.8. Protein concentration was determined by the Bradford assay with albumin (ALB) as a standard. Samples (90 μg) were incubated with α -human ALB-coupled magnetic beads (90 μL , Millipore) and depletion was performed according to the manufacturer’s protocol. Samples were reduced, alkylated and digested overnight at 37°C with sequencing-grade trypsin (1:50, w/w, trypsin/protein; Promega). Tryptic digests were mixed with acetic acid (1:1, v/v) and subjected to solid-phase extraction on a C18 column (HLB, 1 mL; Waters Corp.) according to the manufacturer’s protocol. Fractions containing peptides were dried under vacuum and resuspended in 0.3% formic acid, 5% acetonitrile (0.4 mg mL^{-1}) for LC–MS/MS analysis.

2.7. Liquid chromatography–electrospray ionisation–tandem mass spectrometry (LC–ESI–MS/MS)

Tryptic digests (1.5 μg) were loaded directly onto a 2-cm C18 trap column (packed in-house), washed with 10 μL of solvent A (5% acetonitrile, 0.1% formic acid) and eluted on a 15-cm-long, 75 μM reverse phase capillary column (ProteoPep™ II C18, 300 Å, 5 μm size, New Objective, Woburn, MA, USA). Peptides were separated at 300 nL min^{-1} over a 180-min linear gradient from 5% to 35% buffer B (95% acetonitrile, 0.1% formic acid) on a Proxeon Easy n-LC II (Thermo Scientific, San Jose, CA, USA). Mass spectra were acquired in the positive ion mode, using electrospray ionisation and a linear ion trap mass spectrometer (LTQ Orbitrap Velos®, Thermo Scientific, San Jose, CA, USA). The mass spectrometer was operated in data-dependent mode, and for each MS1 precursor ion scan, the 10 most intense ions were selected from fragmentation by collision-induced dissociation (CID). Other parameters for MS analysis included the following: resolution of MS1 was set at 60,000, normalised collision energy 35%, activation time 10 ms, isolation width 1.5 and the +1 and +4 and higher charge states were rejected.

2.8. Peptide and protein identification

MS/MS spectra were searched against the International Protein Index human (v3.87, 91,464 entries) primary sequence database [21] using Sorcerer™-SEQUEST® (version v. 3.5) (Sage-N Research, Milpitas, CA, USA). Search parameters included trypsin specificity (after Arg or Lys) with up to two missed cleavages. SEQUEST® was searched with a parent ion tolerance of 50 ppm and a fragment ion mass tolerance of 1 amu with fixed Cys alkylation and variable Met oxidation. SEQUEST results were further validated with

PeptideProphet [22] and ProteinProphet [23] using an adjusted probability of ≥ 0.90 for peptides and ≥ 0.96 for proteins. Search results were further processed by the Computational Proteomics Analysis System (CPAS) [24] prior to statistical analysis (see below). Proteins considered for analysis had to be identified in at least 70% of individuals in at least one patient group (e.g., healthy girls, or boys with OSA). When MS/MS spectra could not differentiate between protein isoforms, all were included in the analysis.

2.9. Protein quantification and statistical analysis

Proteins detected by LC–MS/MS were quantified by spectral counting (the total number of MS/MS spectra detected for a protein [25]). Differences in relative protein abundance were assessed with the *t*-test and *G*-test [19,26,27]. Permutation analysis was used to empirically estimate the false discovery rate (FDR) [28]. Significance cut-off values for the *G*-statistic and *t*-test were determined using PepC [29], a software package that maximises the number of differentially abundant proteins identified for a given FDR.

2.10. Elisa

Urine samples were thawed rapidly at 37 °C and clarified by centrifugation at 500g for 10 min. Protein levels in resultant supernatants were quantified using commercially available ELISAs for dipeptidyl peptidase 4 (DPP4) (Abnova; KA0141), AZGP1 (Abnova; KA1689), C-peptide (CP) (Assaypro; EC4101-1), haemopexin (HPX) (Innovative Research, Inc.; IRKTAH2562) and creatinine (Abcam; ab65340) according to the manufacturer's protocols. All protein levels were standardised to urine creatinine levels [30] and statistical significance between the groups was assessed by a two-tailed, Student's *t*-test.

2.11. Functional annotation

Functional enrichments in gene ontology annotations in the urine proteome or differentially abundant putative urine biomarkers (relative to the entire human genome) were identified using the Bingo 2.0 plugin in Cytoscape (V2.8.2) [31]. Statistical significance was assessed using the hypergeometric test ($p < 0.05$) with Benjamini–Hochberg correction [28], and functional categories with ≥ 5 proteins were considered.

3. Results

3.1. Proteomics workflow for urine biomarker discovery

Biomarker discovery strategies based on proteomics are complicated by low protein concentrations and high levels of interfering substances (e.g., salts and nitrogenous bases) in urine. To develop a rigorous and reproducible workflow for proteomics analysis of urine, we developed a four-step procedure involving: (i) centrifugation to remove particulate material and urinary sediment, (ii) depletion of IgG and ALB to facilitate deeper proteome coverage, (iii) protein precipitation to concentrate urine proteins and remove interfering substances and (iv) mass spectrometric analysis by LC–MS/MS (Fig. 1a).

ALB and IgG are highly abundant urine proteins (40–60% of total urinary protein) that interfere with detection of low-abundance species and complicate quantification in label-free proteomic approaches [32]. Magnetic beads were carefully titrated to maximise depletion of ALB and IgG (Fig. 1b and c) and minimise non-specific loss of unrelated proteins, as assessed by loss of serotransferrin (TRF) levels (Fig. 1c). As proteins are more efficiently precipitated in concentrated solutions (due to molecular crowding), we

depleted ALB after protein precipitation. However, IgG depletion was incompatible with the buffer (0.1% RapiGest) used to solubilise protein pellets, and was therefore performed prior to precipitation.

Protein precipitation is a crucial step in urine proteomic studies, which concentrates urinary proteins and removes interfering substances that prohibit direct analysis by MS. For this purpose, we incorporated a method involving TCA/DOC [19,20] because it is well suited for precipitating proteins out of dilute solutions. The reproducibility of this method within and across samples was interrogated by precipitating six aliquots of the same urine sample collected from each of the 10 subjects. This approach yielded highly reproducible results (6% coefficient of variation (CV), intra-sample) over a wide range of urinary protein concentrations (Fig. 1d).

To test the reproducibility of our proteomics workflow, urine samples from 27 children (Table 1) were processed and subjected to LC–MS/MS analysis (Table S1). Based on a minimum of two unique peptide identifications per protein, our approach reliably identified 505 ± 10 proteins per sample. Moreover, variation in sample depth, the number of high-quality peptide identifications per run, was minimal ($10,053 \pm 237$ peptides) indicating that our method was robust and reproducible. Indeed, gene ontology analysis of the detected urinary proteome identified significant enrichments in functional annotations (Fig. 1e) consistent with previous proteomics analyses of urine [33,34].

3.2. Gender and diurnal effects introduce variability into the urine proteome of healthy children

Identifying biomarkers of disease is complicated by the inherent variability in the urine proteome. Gender and diurnal effects represent two important factors that can potentially introduce noise into the measurement. To investigate their impact on the urine proteome under physiological conditions, we collected morning and bedtime samples from healthy boys ($N = 7$) and girls ($N = 6$). Healthy children (ages 2–12 years) were selected by *a priori* excluding participants with genetic or craniofacial syndromes, diabetes, cystic fibrosis or cancer. Additional exclusion criteria included chronic use of medications, steroids or immunotherapy drugs.

Samples were processed through our proteomics workflow (see Fig. 1) and subjected to LC–MS/MS analysis. Proteins were quantified by spectral counting [25], and statistically significant changes in protein levels were identified using a combination of the *t*-test and *G*-test [19,26,27] using cut-offs that minimised the FDR [28,29]. A representative analysis is provided in Fig. 2a, which demonstrates the detection of gender-regulated proteins in morning urine samples upon application of our stringent statistical criteria (*G*-test: $G \geq 1.5$ or $G \leq -1.5$; *t*-test: $\alpha = 0.05$; FDR < 0.05).

Using this approach, we detected substantial differences in the urinary proteome of healthy boys and girls, both in morning ($\sim 7\%$; 50 of 750 proteins) and bedtime (8%; 41 of 750) samples (Fig. 2a and b and Table S2). Interestingly, we observed poor overlap ($< 10\%$) between differentially abundant proteins in morning and bedtime samples, suggesting that gender-related differences were also highly sensitive to diurnal effects (Fig. 2b). For example, TRF levels were elevated in girls at bedtime, while islet cell regeneration factor (REG1A) was specifically increased in morning urine samples collected from boys (Fig. 2c).

In general, urine protein composition was more substantially influenced by gender (69 proteins: *G*-test, $G \geq 1.5$ or $G \leq -1.5$; *t*-test, $p < 0.05$; FDR < 0.05) over diurnal effects (eight proteins: *G*-test, $G \geq 1.5$ or $G \leq -1.5$; *t*-test, $p < 0.05$; FDR < 0.05), an observation that is quite remarkable given that our analysis focussed on prepubescent children. Consistent with this finding, gene ontology analysis of the gender-regulated urinary proteome in healthy

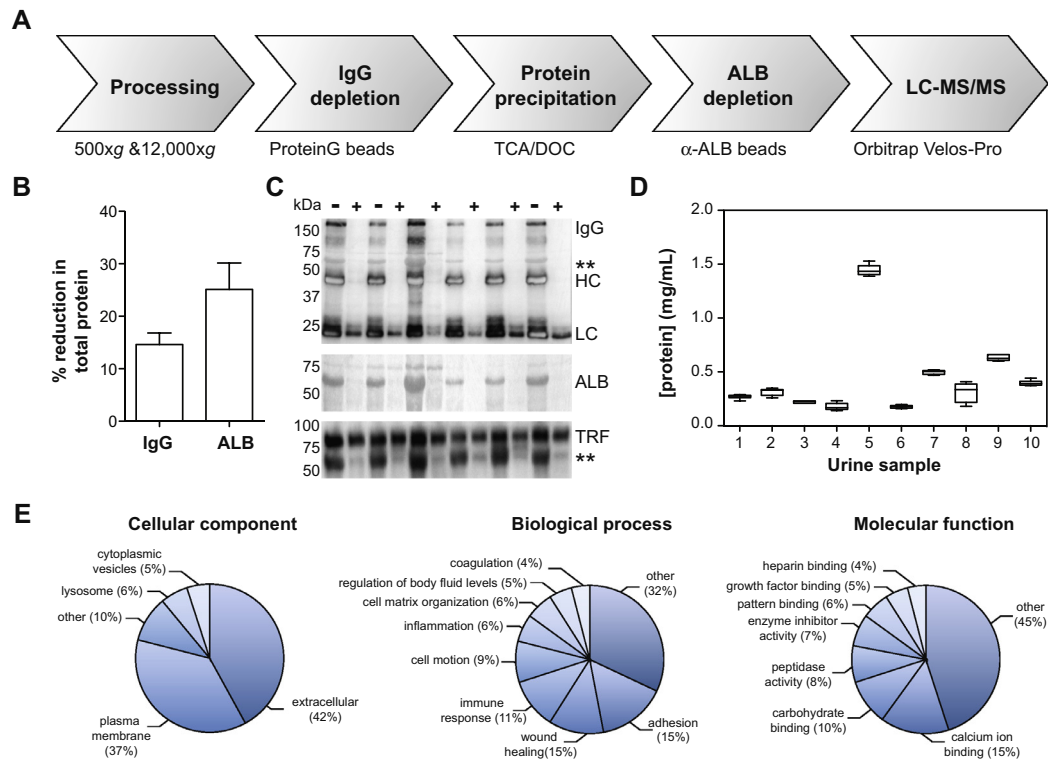


Fig. 1. Pipeline for urine biomarker discovery by LC-MS/MS. *Panel A:* a workflow for proteomic analysis of urine. *Panels B–C* immunoglobulin (IgG) and albumin (ALB) depletion. The extent of depletion of total protein (% reduction) was quantified by Bradford (*Panel B*) and visualised by SDS-PAGE (*Panel C*). Specificity of IgG and ALB removal was assessed by comparing serotransferrin (TRF) levels in depleted (+) and non-depleted (–) samples (*Panel C*). IgG, whole antibody; HC, heavy chain; LC, light chain; **, non-specific detection of ALB. *Panel D:* urine samples were precipitated with TCA/DOC and protein levels were determined for 10 subjects. Results ($N = 6/\text{subject}$) are displayed as box-and-whisker plots (5–95% confidence intervals). *Panel E:* gene ontology analysis of all urine proteins detected by mass spectrometry. All functional annotations presented are statistically significant ($p < 0.05$) based on the hypergeometric test with Benjamini–Hochberg correction.

Table 1
Demographic and polysomnographic characteristics of subjects.

	Control ($N = 13$)	OSA ($N = 14$)	t -Test (p -value)
Age (years)	7.5 ± 0.8	5.9 ± 0.6	0.11
Gender (boy, girl)	7,6	7,7	N/A
BMI, z -score	0.6 ± 0.3	1.2 ± 0.5	0.27
AHI (events/hr/total sleep time)	0.4 ± 0.1	23.3 ± 5.3	0.0002

Abbreviations: BMI = body mass index; AHI = obstructive apnoea–hypopnoea index; OSA = obstructive sleep apnoea. Where applicable, results are presented as means \pm SEM.

children revealed significant enrichments in functional annotations that are not classically associated with gender (cell adhesion, $p = 6.0 \times 10^{-7}$; pattern binding, $p = 7.0 \times 10^{-3}$; complement and coagulation cascades, $p = 4.2 \times 10^{-3}$). In sharp contrast, this approach failed to identify significance in more intuitive modules such as female pregnancy ($p = 0.11$) or embryo implantation ($p = 0.11$).

3.3. Gender and diurnal dependence of urine biomarker discovery of paediatric OSA

Having demonstrated that gender and diurnal factors substantially regulate the urinary proteome of children under physiological conditions, we sought to investigate how these differences would impact the process of biomarker discovery among children with OSA. Children (ages 2–12 years) with moderate to severe OSA, as assessed by the PSG-derived criterion of AHI (AHI > 5 events per hour total sleep time), were recruited along with age- and gender-matched controls. Their demographic characteristics

were such that no statistically significant differences in age, gender, ethnicity or BMI distribution were present (Table 1).

Using stringent criteria for quality and reproducibility of protein detection (see Section 2), our mass spectrometric analyses of urine samples identified 742 urine proteins across all patient samples (Table S1). To investigate the impact of gender and diurnal variation on biomarker discovery, we performed statistical analysis (using the t -test and G-test [19,27,29]) in three ways (Fig. 3a). In level 1 analysis, protein levels were averaged across morning and bedtime samples and groups were not differentiated according to gender. Level 2 analysis investigated morning and bedtime samples independently, while level 3 analysis treated samples in a collection time- and gender-dependent fashion (Fig. 3a).

Six candidate biomarkers of paediatric OSA were identified in level 1 analysis (Table S3). Notably, orosomucoid 1 (ORM1), a protein that was initially identified in our previous OSA biomarker screen [15], was also detected in this analysis. The statistical significance level for ORM1, however, barely cleared statistical thresholds, and subsequent ELISA measurements failed to validate this finding (control: $42.8 \pm 14.3 \mu\text{g mg}^{-1}$ creatinine; OSA:

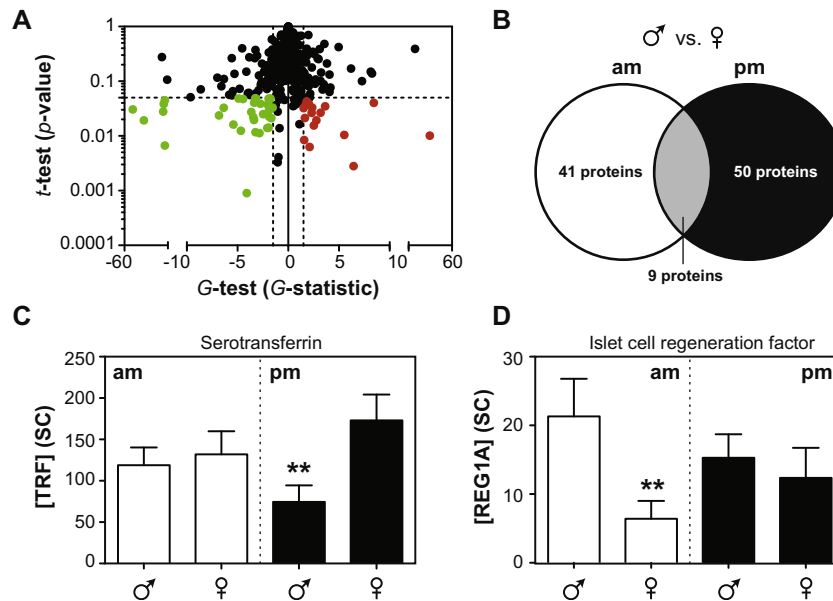


Fig. 2. Gender and diurnal effects on the urinary proteome of healthy children. Morning (am) and bedtime (pm) urine samples were collected from healthy boys ($N = 7$) and girls ($N = 6$) and subjected to LC–ESI–MS/MS analysis. Proteins were quantified by spectral counting and differentially abundant proteins were detected using the t -test and G-test. **Panel A:** A representative statistical analysis demonstrating proteomic differences in morning samples between boys and girls. Red, elevated in boys; green, reduced in boys. Confidence intervals (dashed lines; $G > 1.5$ or $G < -1.5$ and $\alpha = 0.05$) and the FDR ($< 5\%$) were established by permutation analysis. Proteins that were elevated in boys were assigned negative values in the G-test. **Panel B:** A comparison of differentially abundant proteins in boys (relative to girls) in morning and bedtime samples. **Panels C–D:** examples of proteins (TRF and REG1A) that are subjected to both gender and diurnal regulation. Results are means \pm SEMs; statistical significance (**) was assessed by a combination of the t -test and G-test.

$68.5 \pm 29.5 \mu\text{g mg}^{-1}$ creatinine, $p = 0.37$, $N = 14$). A substantial increase in the number of biomarkers detected was evident when morning and bedtime samples were treated independently (level 2, 45 proteins) and a further, more dramatic, increase was visualised when gender was also accounted for in the analysis (level 3, 192 proteins) (Fig. 3a, Table S3). Accounting for gender and sampling time also increased the statistical significance of the candidate biomarkers identified. In this regard, 26% of the candidate biomarkers exhibited $p < 0.005$ (t -test) in level 3 analysis, while no proteins satisfied this criterion in level 2 or level 1 analysis. Moreover, fatty acid-binding protein 3 (FABP3), a candidate biomarker that cleared statistical thresholds in all levels of analysis, demonstrated substantially improved statistics from level 1 (G-test: $G = 2.4$; t -test, $p = 0.022$) to level 2 (G-test: $G = 6.0$; t -test, $p = 0.006$) to level 3 analysis (G-test: $G = 12.8$; t -test, $p = 0.0009$).

In general, morning urine samples were overrepresented in differentially abundant proteins, a result largely based on the overwhelming effect of OSA on the urinary proteome of boys (Fig. 3b). This observation is not surprising given that OSA is a sleep disorder characterised by repetitive respiratory events at night that should therefore be more likely to manifest in morning urine; however, the opposite results emerged among girls, in whom bedtime urine samples yielded a higher number of candidate biomarkers (Fig. 3b). Moreover, differentially abundant proteins were highly specific for gender and sampling time, as poor overlap ($\sim 3\%$) was observed in the candidate biomarkers identified in boys and girls across morning and bedtime samples (Table S3). Importantly, gender differences in the biomarkers detected could not be accounted for by differences in age, disease severity or obesity (BMI z-score) as these parameters were not significantly different between the groups (Fig. 3c).

Taken together, our results suggest that failing to account for sampling time and gender substantially masks significant differences in protein abundance associated with a disease state such as OSA. This concept is clearly illustrated by global proteomic analysis of morning urine samples with the t -test and G-test, which

shows dramatic improvements in both number and statistical significance of biomarkers identified (Fig. 3d). Similar conclusions emerge at the individual protein level using DPP4 as an example (Fig. 3e).

3.4. Validation of candidate biomarkers identified by proteomic analysis

To validate our findings, we used commercially available ELISA assays to measure urinary levels of four candidate biomarkers. As protein levels in urine are highly variable, and influenced by body fluid volume, all measurements were standardised against corresponding urinary creatinine levels [30]. ELISA measurements generally correlated well with label-free quantification by MS/MS (e.g., HPX, $p < 0.0001$, $R^2 = 0.52$; Fig. 4a) and provided strong validation for gender and diurnal regulation of protein levels (e.g., DPP4; compare Figs. 3d and 4b). Gender and diurnal effects on protein levels were not due to systematic biases in creatinine levels, which were insensitive to gender and time of collection (Fig. S1). In total, ELISA assays provided independent confirmations of changes in protein levels for four candidate biomarkers detected in our proteomic analyses: DPP4 ($p = 0.02$), HPX ($p = 0.02$) and CP ($p = 0.01$) emerged as reliable indicators of OSA in boys, and AZGP1 ($p = 0.07$) was identified in girls (Fig. 4b and c). Moreover, because ELISA assays involved minimal processing of urinary samples (centrifugation), while proteomic analyses required substantial processing efforts (centrifugation, IgG and ALB depletion, protein precipitation, sample digestion, etc.), the strong concordance between these two approaches further suggests that our proteomic workflow for urine biomarker discovery is robust.

3.5. Biomarkers of paediatric OSA map to pathophysiological functional modules

Having identified a wide range of candidate biomarkers in urine collected from children with OSA, we next sought to determine

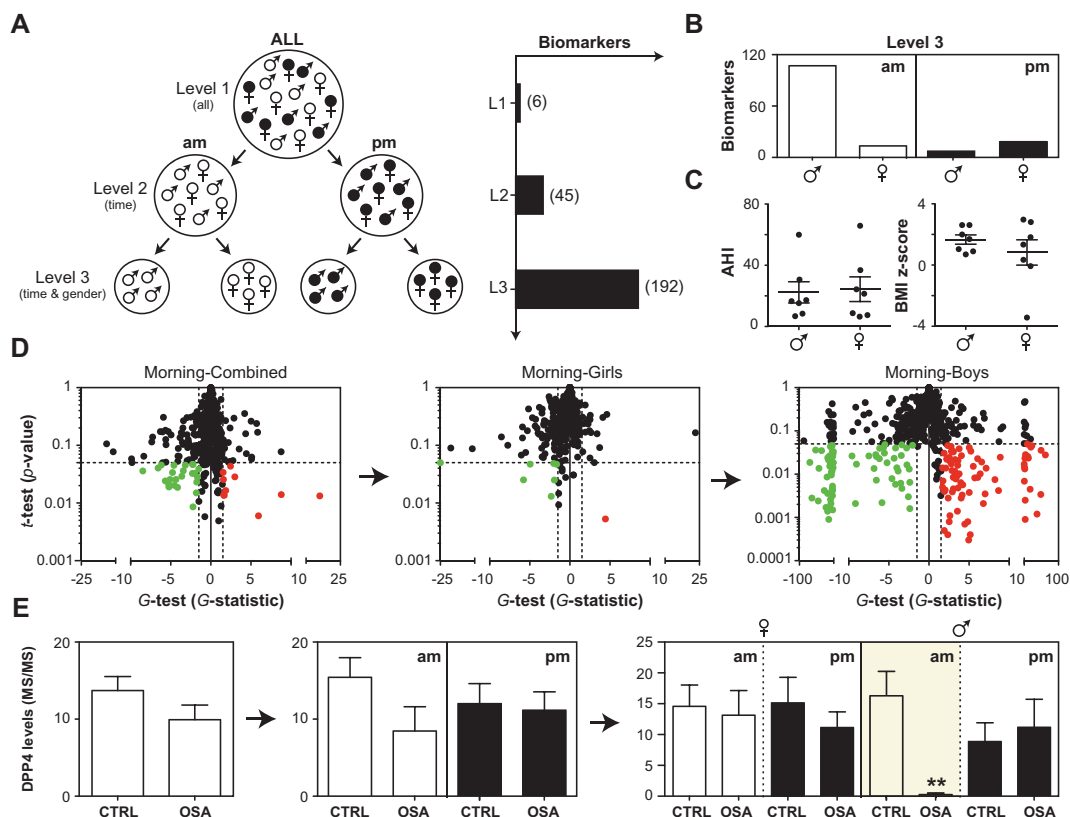


Fig. 3. Identification of candidate biomarkers of paediatric OSA. Morning (am) and bedtime (pm) samples were collected from children with and without OSA and subjected to LC-MS/MS. *Panel A:* analysis of proteomic data was performed as follows: level 1 (L1), morning and bedtime measurements were averaged and boys and girls were pooled; level 2 (L2), analyses for morning and bedtime samples were conducted independently; level 3 (L3) analyses for morning and bedtime samples were conducted independently in both boys and girls. The number of candidate biomarkers identified at each level is shown in parentheses. *Panel B:* Biomarkers detected in level 3 were split according to collection time and gender. *Panel C:* AHI and BMI z-scores in boys and girls with OSA. *Panel D:* a demonstration of the “gender effect” on global proteomic analysis (based on the *t*-test and *G*-test) of morning urine samples. Red, up-regulated in OSA; green, down-regulated in OSA; dashed lines confidence intervals (FDR < 5%). *Panel E:* dipeptidyl peptidase 4 (DPP4) as an example of a specific biomarker for OSA in the morning samples of boys. Protein levels (mean ± SEMs) were determined by spectral counting. **, statistically significant based on the *t*-test and *G*-test.

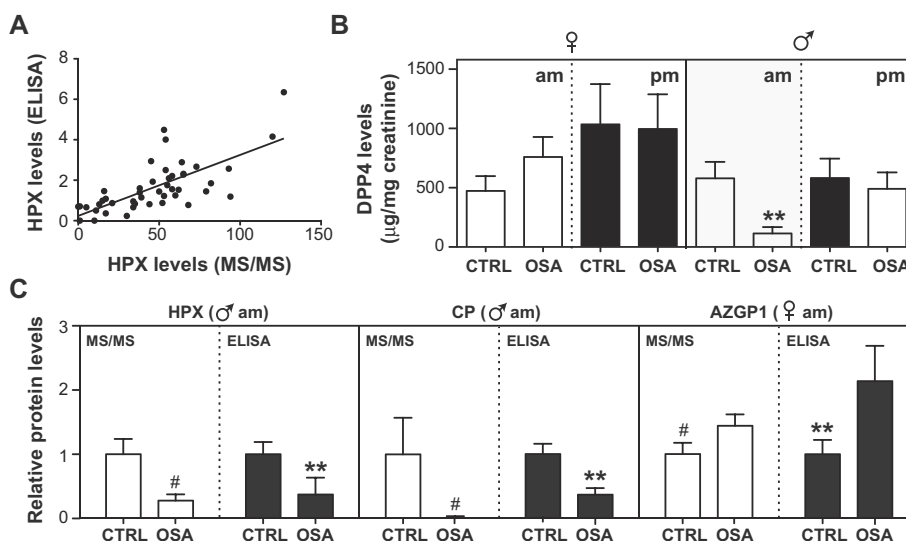


Fig. 4. Validation of mass spectrometry data by ELISA. Differentially abundant proteins identified by proteomic analysis were validated in morning (am) and bedtime (pm) samples using commercially available ELISA assays. *Panel A:* Comparison of haemopexin (HPX) level quantified by mass spectrometry (MS/MS) and ELISA (ng/mg creatinine). Linear regression analysis (line) detected a strong positive correlation ($R^2 = 0.52$, $p < 0.0001$) between both techniques. *Panel B:* measurement of DPP4 levels by ELISA demonstrating specific reduction of dipeptidyl peptidase 4 (DPP4) in morning urine samples (compare to Fig. 3d). *Panel C:* comparison of HPX (ng/mg creatinine), ceruloplasmin (CP; ng/mg creatinine) and zinc-α-2-glycoprotein (AZGP1; ng/mg creatinine) levels quantified by MS/MS and ELISA. Measurements were normalised relative to control samples. Where applicable, results are means ± SEMs. #, statistically significant based on the *t*-test ($p < 0.05$) and *G*-test ($G > 1.5$). **, statistically significant based on the *t*-test ($p < 0.05$).

whether those proteins mapped to specific functional pathways. To this end, we used gene ontology analysis to organise the 192 proteins into functional modules based on biological processes and molecular function (Fig. 5). This strategy identified significant enrichment (relative to the entire human genome) in a number of functional annotations including acute-phase proteins ($p = 8.4 \times 10^{-5}$), angiogenesis ($p = 2.7 \times 10^{-3}$), haemostasis ($p = 4.2 \times 10^{-8}$), leucocyte immunity ($p = 2.4 \times 10^{-2}$) and lipid binding ($p = 2.3 \times 10^{-4}$). Previous studies provide evidence that all of these pathways are affected in OSA. For example, disruption in inflammatory/immune, lipid, angiogenic and haemostatic pathways have all been reported in patients with OSA [35–38], and are proposed as the mechanistic basis for the heightened prevalence of associated comorbidities in OSA, such as obesity, diabetes and atherosclerosis. Taken together, these findings suggest that aside from providing an abundant repository of disease biomarkers, the urinary proteome also comprises a wealth of information concerning disease-related pathological processes.

4. Discussion

Using a rigorous and reproducible workflow for proteomic analysis, we here demonstrate that gender and diurnal effects introduce significant variability into the urinary proteome of healthy children. By incorporating these constitutive determinants of variance into our analyses, 192 candidate biomarkers were *a priori* identified in urine collected from children with OSA. Moreover, we show that most if not all (~97%) of these biomarkers retained their predictive ability only if their use was implemented in the contextual setting of their collection (i.e., morning in boys, or bedtime in girls), a result that was validated by ELISA measurements. These results highlight the complexity of the biomarker discovery process and suggest that carefully contextualised biomarker discovery strategies will be obligatorily needed to effectively detect human disease across broad populations.

Genetic and environmental perturbations impose dramatic variability on protein expression patterns in individuals. Epigenetic, transcriptomic, metabolomic and proteomic studies have highlighted the dynamics of regulation of gene expression within healthy populations [37,39]. For example, DNA methylation patterns in healthy human tissues were highly sensitive to age and environmental factors [39]. Similarly, metabolites relating to mitochondrial energy metabolism were found to differentiate gender and age in healthy adults [37]. Current findings demonstrating that diurnal- and gender-related effects operate as powerful modulators of the urinary proteome in healthy children are highly consistent with these other studies.

Given the inherent variability in healthy populations, 'how does this influence how we detect disease'? Traditional approaches to biomarker discovery are based on the premise that introducing a

complex pathophysiological process will overwhelm the substantial underlying heterogeneity, and produce common factors or signatures that can be leveraged to detect disease in broad populations. Our findings suggest an alternative scenario, whereby disease predictably exaggerates the underlying variability, resulting in the identification of biomarkers that are effective only in a context-specific manner. Indeed, we show that gender and diurnal differences in urinary proteome are more robust in children with OSA than in healthy controls.

Importantly, our identification of gender-related biomarkers of OSA cannot be attributed to disease aetiology. To our knowledge, there are no previous studies documenting distinct pathophysiological responses or clinical outcomes of OSA in boys and girls. Although a diurnal effect in OSA is predictable, as OSA is a sleep disorder, this rationale cannot explain why such a strong diurnal regulation was observed only in boys, or why diurnal factors determined the gender-related effects on urine composition in healthy children. Based on these arguments, we believe that our findings can be extrapolated to a broad range of diseases, and may further provide the basis for retrospectively incorporating these factors in the context of a systematic reassessment of pre-existing biomarker discovery data sets.

Rigorous matching of cases and controls is a precaution often utilised so as to minimise the contribution of 'noise-related confounders' that could affect urine protein composition. Although somewhat counterintuitive, our data suggest that such noise-reducing strategies may actually have the opposite effect. Pooling urine samples within an individual patient, or binning information across genders, substantially interfered with our ability to reliably detect disease. Accordingly, we demonstrate dramatic improvements in the statistical significance and quantity (~30-fold increase) of OSA biomarkers identified when the 'noise-related factors' are incorporated into the biomarker discovery process.

It is important to stress that the conceptual innovations introduced herein were obtained in 'children', a population that is substantially protected from additional environmental and pathological confounders (e.g., puberty, smoking, chronic illness, medications, etc.) whose incidence and heterogeneity increase exponentially with age. Accordingly, extrapolating these concepts to the detection of disease biomarkers in adult populations will likely require the incorporation of more complex contextualised schema into the biomarker discovery process. In general, these methodological approaches underlie the foundation for implementation of personalised medicine, which could potentially revolutionise the way we diagnose and treat disease. Although the conceptual framework of personalised biomarkers for disease detection has only been recently advanced, it is rapidly gaining traction [40–42]. For example, a recent study leveraged massive parallel sequencing to develop personalised biomarkers capable of identifying translocations in solid tumours [43].

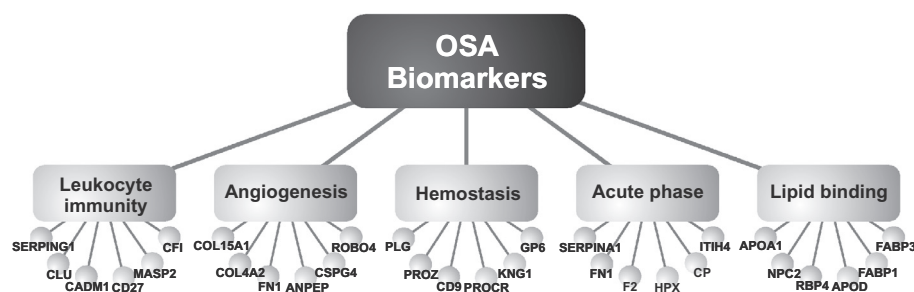


Fig. 5. Biomarkers of paediatric OSA map to pathophysiological modules. Gene ontology analysis of the 192 candidate biomarkers identified numerous functional modules enriched in children with OSA ($p < 0.05$, hypergeometric test with Benjamini–Hochberg correction). Six representative proteins in each functional module are presented as examples.

At this time, the physiological and pathophysiological mechanisms mediating gender and diurnal regulation of the urinary proteome in children with OSA remain elusive. However, based on our functional assessment of the proteomic data by gene ontology analysis, it becomes apparent that urine adequately reflects pathological processes of the disease. Inflammatory, immune, lipid, haemostatic and angiogenic pathways are well-recognised clinical sequelae of OSA and its associated co-morbidities [35–38]. It should be noted that bioinformatic analysis of urine proteins should be interpreted with caution as changes in urine protein levels are not necessarily correlated to blood levels.

A comparison of our list of candidate biomarkers identified in children with OSA to those previously reported for adults with diabetes [7], coronary artery disease [8] and chronic obstructive pulmonary disease [44] revealed intriguing overlaps. These findings suggest that by targeting similar proteins and/or biological pathways, OSA may predispose children to develop these associated disorders later on in life. Given the large changes observed in the morning urine proteome of boys with OSA, we would further predict that boys might be at a greater risk to develop such co-morbidities. However, future validation of such hypotheses in large-scale cohorts appears warranted. OSA is a highly prevalent health problem in children, particularly among obese children. Considering the escalating problem of childhood obesity in the United States and around the world, properly and timely diagnosing and treating OSA will continue to pose a significant challenge due to the onerous nature of PSG and the scarce availability of paediatric sleep experts. Our identification of candidate urinary biomarkers of OSA in children may enable simplified and cost-effective diagnostic algorithms [45], and perhaps lay the foundation for new approaches to monitoring treatment efficacy for this important medical problem.

Conflict of interest

The ICMJE Uniform Disclosure Form for Potential Conflicts of Interest associated with this article can be viewed by clicking on the following link: <http://dx.doi.org/10.1016/j.sleep.2014.01.010>.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.sleep.2014.01.010>.

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